A high concentration of DMSO activates caspase-1 by increasing the cell membrane permeability of potassium

Yang Xiang · Ming-ming Zhao · Sujiao Sun · Xiao-Long Guo · Qiquan Wang · Sheng-An Li · Wen-Hui Lee · Yun Zhang

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Abstract Dimethyl sulfoxide (DMSO) is widely used in the laboratory and in clinical situations because it is soluble in both aqueous and organic media and can be used to treat many types of diseases. Thus, it is meaningful to assess the comprehensive and in-depth biological activities of DMSO. Here, we showed that a high concentration of DMSO induced pro-inflammatory cytokine interleukin-1 β (IL-1 β)

secretion from the monocytic cell line THP-1. DMSO-induced IL-1 β secretion was dependent on intracellular caspase-1 activation. Further study revealed that the activation of caspase-1 by DMSO relied on NLRP3 inflammasome formation. It is generally accepted that the NLRP3 inflammasome is activated by reactive oxygen species generation or potassium efflux; however, the common NLRP3 inflammasome trigger remains controversial. Here, we showed that although DMSO is a ROS scavenger, this chemical increases membrane permeability and potassium efflux, and the formation of the NLRP3 inflammasome reflects the increased membrane permeability and potassium efflux induced by DMSO. The present study reveals a new characteristic of DMSO, which should be considered when using this chemical in either the laboratory or the clinic.

Yang Xiang, Ming-ming Zhao and Sujiao Sun these authors have contributed equally to this work.

Y. Xiang
Human Aging Research Institute and School of Life Sciences, Nanchang University, Nanchang, China

Y. Xiang (✉) · M. Zhao · X.-L. Guo ·
Q. Wang · S.-A. Li · W.-H. Lee · Y. Zhang (✉)
Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China
e-mail: xiangyang@ncu.edu.cn;
yang_xiang000@hotmail.com

Y. Zhang
e-mail: zhangy@mail.kiz.ac.cn

S. Sun
Medical Cosmetology Teaching and Research Section, Dali University School of Clinical Medicine, Jiashibo Road 32, Dali, Yunnan Province 671000, China

M. Zhao · Q. Wang
Kunming College of Life Science, University of Chinese Academy of Sciences, Beijing 100049, China

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Introduction

Dimethyl sulfoxide (DMSO), possessing a highly polar domain and two apolar groups, is soluble in both aqueous and organic media. This characteristic makes DMSO an excellent solvent for many chemicals in biological studies, and DMSO can rapidly penetrate the plasma membrane, making this chemical a useful

carrier for drugs and a cryoprotective agent. DMSO is commonly used as a solvent for the *in vivo* administration of several water-insoluble substances. This chemical has also been used in the treatment of dermatological (Hsieh et al. 1987), urinary (McCammon et al. 1998), pulmonary (Iwasaki et al. 1994) and renal amyloidosis (Murav'ev et al. 1990). DMSO has reactive oxygen species (ROS) scavenging properties. Thus, DMSO has been proposed for the treatment of several inflammatory diseases (Santos et al. 2003). However, the toxicity and side effects of DMSO have also been widely reported (Davis et al. 1990). Cellular and molecular studies of DMSO have revealed that this chemical induces the release of K^+ , Ca^{2+} , and lactate dehydrogenase from the intracellular space into the extracellular space during the cryopreservation of human platelets (Bock et al. 1995). In addition, DMSO induces the apoptosis of EL-4 lymphoma cells (Liu et al. 2001).

NLRP family proteins are important components of inflammasomes. Inflammasomes are molecular platforms that assemble through the hetero-oligomerization of NLRP, ASC and pro-caspase-1. Upon assembly of this macro-molecular platform, pro-caspase-1 is activated and subsequently processes pro-inflammatory cytokines, such as IL-1 β and IL-18 (Schroder and Tschopp 2010). Apart from a pivotal role in the host immune response to pathogens, including fungi (Hise et al. 2009), bacteria and parasites (Shio et al. 2009), inflammasomes are also the first line of response to non-infectious cell stresses (Ogura et al. 2006), including extracellular adenosine triphosphate (ATP) and stimuli increased membrane permeability. Until recently, extensive studies of the types of inflammasomes included the NLRP3 inflammasome, NLRC4 inflammasome and AIM2 inflammasome (Schroder and Tschopp 2010). Among these structures, the NLRP3 inflammasome may be the most attractive type, as this complex is activated by a wide range of stimuli, including pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular patterns (DAMPs). After the activation of caspase-1 following inflammasome assembly, the proteins are unconventionally secreted from the cell, including but not limited to IL-1 β , interleukin-18 and interleukin-33 (Keller et al. 2008).

Evaluating the biological effect of DMSO is meaningful to improve its laboratory and clinical use. Considering the characteristics of increasing cell

membrane permeability, it is reasonable to infer that DMSO could activate the NLRP3 inflammasome. In the present study, we assessed whether DMSO activates the inflammasome and determined the mechanism underlying this effect.

Materials and methods

Cell culture

THP-1 cells obtained from the Kunming Cell Bank, Chinese Academy of Sciences (Kunming, Yunnan, China) were grown in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 U/mL) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), streptomycin (100 μ g/mL) (Sigma-Aldrich) and 2 mM L-glutamine (Sigma-Aldrich) at 37 °C. Before use, the cells were treated with 50 ng/mL PMA (Sigma-Aldrich) for 6 h, and then the medium was replaced with serum free RPMI 1640 medium, followed by further incubation for an additional 8 h.

Cytokine determination

Cytokine determination was performed as previously described (Xiang et al. 2013). THP-1 cells were cultured in 12-well plates and subsequently differentiated with PMA. The cells were stimulated with DMSO (Sigma-Aldrich) for the indicated time periods. The supernatants were harvested and centrifuged at 11,000 $\times g$ for 5 min. Subsequently, the IL-1 β concentration was assayed using a commercial ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Reverse transcription polymerase chain reaction (RT-PCR)

The cells were treated with different concentrations of DMSO or LPS for 2 h, and subsequently, the cells were washed 2 times with phosphate-buffered saline. Total RNA was extracted using the Total RNA Extraction Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. First-strand cDNA was synthesized using the *PrimeScript* 1st strand

cDNA Synthesis Kit (Takara, Kusatsu, Shiga, Japan). Then, IL-1 β and β -actin were amplified.

Western blotting

Cells were grown in 12-well plates and were treated with DMSO for the indicated time periods. The cells were subsequently collected, washed, and immediately lysed on ice in lysis buffer containing 50 mM HEPES (pH 7.4), 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 5 mg/mL aprotinin (Sigma-Aldrich), 5 mg/mL leupeptin (Sigma-Aldrich) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). The proteins (30 μ g) were electrophoresed on SDS–polyacrylamide gels and transferred onto PVDF membranes. The membranes were subsequently blocked with 3% BSA (Beyotime, Jiangsu, China) and incubated with the appropriate primary (Caspase-1 p20, sc-398715, Santa Cruze Biotechnology, Santa Cruze, CA, USA) and secondary antibodies (donkey anti-goat IgG-HRP, Sc-2020). The protein bands were visualized using Super Signal chemiluminescence reagents (Pierce, Rockford, IL, USA), as previously described (Xiang et al. 2012).

Caspase-1 activity assay

Caspase-1 activity was measured using a Caspase-1/ICE Colorimetric Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions (Peeters et al. 2013; Joshi et al. 2002).

RNA interference

siRNA targeting NLRP3 (sc-45470), NLRC4 (sc-60329), ASC (sc-37282) and caspase-1 (sc-29922) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The siRNA was transfected with Hiperfect (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

Reactive oxygen species (ROS) assessment

After stimulation, for the estimation of intracellular ROS, the supernatant was replaced with DCFH-DA (10 μ M) (Sigma) and incubated for 1 h at 37 °C. The wells were then washed with freshly prepared Hank's balanced salt solution, and the fluorescence intensity was measured using a fluorescence microplate reader

with an excitation wavelength of 488 nm and emission wavelength of 525 nm (Alvarez and Munoz-Fernandez 2013).

Potassium efflux assay

Potassium efflux was assayed as previously described (Liu et al. 2008). The cells were treated with different concentrations of DMSO for 2 h. Subsequently, the cells were washed with phosphate-buffered saline and lysed with 3% ultrapure HNO₃. Intracellular K⁺ was then determined using a Thermo iCAP6300 spectrometer.

Statistical analysis

All data are presented as the Mean $\pm$ SD. Two-sample comparisons were performed using Student's *t*-tests.

Results

DMSO induces IL-1 β secretion by PMA-differentiated THP-1 cells

THP-1 cells were differentiated with PMA prior to use. IL-1 β was secreted from PMA-differentiated THP-1 cells after incubation with DMSO for 2 h in a dose-dependent manner (Fig. 1a). At concentrations of 0.5 and 1%, DMSO slightly increased the secretion of IL-1 β . When the concentration of DMSO was higher than 2%, the level of IL-1 β in the culture medium was significantly elevated. No significant differences in the induction of IL-1 β secretion were observed between 2, 5 and 10% DMSO concentrations. When 2% DMSO was added to the culture medium, low-level IL-1 β secretion was detected from 30 min after stimulation. At 2 h after DMSO addition, the level of secreted IL-1 β reached a plateau at approximately 230 pg/mL (Fig. 1b). These results demonstrated that DMSO induced the rapid secretion of IL-1 β .

Caspase-1 is activated by DMSO

Since IL-1 β secretion was induced by DMSO, we next determined how DMSO induces IL-1 β secretion. IL-1 β secretion involves two key steps: (1) production of

Fig. 1 DMSO induced IL-1 β secretion in a dose-dependent manner. **a** PMA-primed THP-1 cells were treated with different concentrations of DMSO, ATP or α -hemolysin for 2 h. Secreted

IL-1 β was quantified using ELISA. **b** PMA-primed THP-1 cells were treated with 2% DMSO for 15–120 min. ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$

a pro-IL-1 β protein in response to Toll-like receptor activation and (2) processing of pro-IL-1 β by cysteine protease, including caspase-1 (Vance 2010). The results demonstrated that the mRNA level of IL-1 β secreted from PMA-differentiated THP-1 cells showed almost no change after incubation with DMSO (0.5, 2 or 10%) for 2 h (Fig. 2a). This finding indicated that DMSO does not enhance the production of pro-IL-1 β , at least within 2 h. Thus, it is reasonable to infer that DMSO may have the ability to facilitate the processing of pro-IL-1 β into mature IL-1 β . After DMSO stimulation, the cells were lysed for western blotting analysis to detect the p20 subunit of caspase-1, which is indicative of the degree of caspase-1 activation. The caspase-1 p20 subunit can be detected after incubation with different concentrations of DMSO for 2 h (Fig. 2b). A colorimetric substrate (Ac-YVAD-pNA) of caspase-1 was also used to detect caspase-1 activation after DMSO stimulation, demonstrating that DMSO enhances the cleavage of this substrate (Fig. 2c).

Caspase-1 activation by DMSO is dependent on the NLRP3 inflammasome

Inflammasomes, such as the NLRP1, NLRC4, NLRP3 and AIM2 inflammasomes, are upstream protein complexes that activate caspase-1. NLRC4, NLRP3

and ASC were knocked down using siRNA to assess the type of inflammasome activated by DMSO. The results showed that knockdown of ASC and NLRP3 attenuated DMSO-induced IL-1 β secretion (Fig. 3a). However, knockdown of NLRC4 had no influence on IL-1 β secretion. This result indicated that DMSO activated caspase-1 through NLRP3 inflammasome activation. RNA interference of NLRP3 inflammasome components also attenuated the activation of caspase-1 by DMSO (Fig. 3b).

Potassium efflux but not ROS generation are involved in NLRP3 inflammasome activation by DMSO

NLRP3 inflammasome activation involves ROS generation (Tschoop and Schroder 2010), potassium efflux (Munoz-Planillo et al. 2013), and lysosome destabilization (Lima et al. 2013). We further investigated how DMSO activates the NLRP3 inflammasome. The influences of DMSO on intracellular ROS were assayed using DCFH-DA, and the results showed that DMSO did not increase the ROS level but rather decreased ROS generation (Fig. 4a); this finding was consistent with the widely accepted characteristics of DMSO as a ROS scavenger. We next assayed whether DMSO influenced intracellular potassium levels, and the results showed that DMSO at concentrations

Fig. 2 DMSO induces the cleavage of caspase-1. **a** THP-1 cells were treated with 0.5–10% DMSO, and the gene expression of pro-IL-1 β was verified using semi-quantitative PCR. **b** PMA-primed THP-1 cells were treated with 0.5–10% DMSO, and caspase-1 cleavage was detected by immunoblotting the caspase-1 p20 subunit. **c** After treatment with different concentrations of DMSO, PMA-primed THP-1 cells were lysed and the caspase-1 activity was detected using the colorimetric substrate YVAD-pNA. Caspase-1 activity was measured based on absorbance at 405 nm. ** $P < 0.01$

between 0.5 and 10% induced potassium efflux (Fig. 4b). Based on these results, we can infer that inflammasome activation induced by DMSO may result from potassium efflux but not ROS generation.

Discussion

DMSO has been identified since the nineteenth century and used for laboratory and clinical purposes since the 1960s. Clinically, DMSO has been widely used as a solvent for many types of drugs, and this

chemical has also been used to treat diseases. The most well-known application of DMSO in the laboratory is as a cryoprotectant and solvent of water-insoluble chemicals. Thus, it is important to explore the characteristics of DMSO, including its toxicity, and the biological activities of DMSO should be considered when using this chemical as a solvent or cryoprotectant.

The potential toxicities have been extensively studied. The reported toxicity of DMSO, including induction of the apoptosis of lymphocytes (Aita et al. 2005) and cells in the developing central nervous system (Hanslick et al. 2009). Regarding the influences of DMSO on the immune system, a previous study reported that DMSO inhibits NF- κ B activation, thus confirming the anti-inflammatory activity of this chemical (Essani et al. 1997). However, other reports have shown that DMSO activates NK and NKT cells (Masson et al. 2008). These results seem somewhat controversial. Thus, in the present study, we examined the immunotoxicity of DMSO in vitro using a monocytic cell line.

The results showed that DMSO indeed has pro-inflammatory activity, as this chemical induces IL-1 β a typical pro-inflammatory cytokine. In fact, a previous study reported that DMSO augments IL-1 β secretion induced by LPS (Xing and Remick 2005). However, the present study showed that DMSO solely induces IL-1 β secretion. We further demonstrated that DMSO primarily facilitates the processing of pro-IL-1 β without enhancing the production of pro-IL-1 β . Caspase-1 and its upstream inflammasomes are the main platform for processing pro-IL-1 β (Martinon et al. 2002), thus promoting the secretion of matured IL-1 β .

Inflammasomes (particularly the NLRP3 inflammasome) are emerging as key regulators of innate and adaptive immunity (Menu and Vince 2011). The NLRP3 inflammasome can be triggered by a variety of stimuli, including pore-forming toxins (Franchi et al. 2012; Xiang et al. 2014; Zhang 2015) and ATP (Lee et al. 2012). NLRP3 inflammasome activation may initiate innate immunity against microbial infection (Joly and Sutterwala 2010). The activation of this inflammasome also regulates adaptive immune system (Chen et al. 2011). However, the excessive activation of the NLRP3 inflammasome is involved in the occurrence of inflammatory diseases (Misawa et al. 2013). During the cryopreservation of cells using 10%

Fig. 3 DMSO activates caspase-1 via induction of NLRP3 inflammasome assembly. PMA-primed THP-1 cells were transfected with siRNA of caspase-1, NLRP3, NLR4 or ASC. The cells were subsequently treated with 2% DMSO for

2 h. The secreted IL-1 β was quantified using ELISA (a), and caspase-1 activation was quantified based on the cleavage of YVAD-pNA (b)

Fig. 4 DMSO reduce the generation of ROS but augmented the efflux of potassium. PMA-primed THP-1 cells were treated with different concentrations of DMSO, ATP or α -hemolysin. ROS

were quantified using DCFH-DA staining (a). Potassium efflux was quantified using a flame spectrometer (b)

DMSO in fetal bovine serum, the cells are generally allowed to keep at room temperature (about 25 °C) for about 5 min to allow DMSO to pass through the cell membrane before the cooling procedure is started. During cell thawing procedure, the cells are also needed to be cultured in a DMSO rich medium (about 1% DMSO) before changing fresh medium. Thus, there is enough time and concentration for DMSO to

activate NLRP3 inflammasome during cell freezing and thawing.

In conclusion, DMSO-induced pro-inflammatory cytokine secretion depends on the NLRP3 inflammasome, and this mechanism involves increased cell membrane permeability and potassium efflux. This characteristic should be considered when using DMSO in the laboratory and clinic.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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